Lethbridge iGEM 2014 Standard Protocols:

Restriction Digest

Reagent	Volume
MilliQ	6 ul
10x Cutsmart Buffer	2 ul
DNA	10 ul
Enzyme 1	1 ul
Enzyme 2	1 ul
Total Volume	20 ul

Digest is put in 37C incubator for 3hrs – overnight. Restriction enzymes heat killed at 80C for 20 minutes

PCR

Standard PCR program:

1 – 98C for 5 minutes – Initial Denature

2 – 98C for 30 seconds – Denature

3 – Annealing temp* for 30 seconds – Annealing

4 – 72C for 1min/Kb (PFU) or 20s/Kb (Phusion) - Extension

5 – 72C for 5 minutes – Final extension

*Annealing temp for Pfu PCRs calculated by subtracting 5C from the Tm of the binding region of the primer. 5C is subtracted from the lowest of the pair of Tms

PFU

*Annealing temp for Pfu PCRs calculated by subtracting 5C from the Tm of the binding region of the primer. 5C is subtracted from the lowest of the pair of Tms

Reagent	Volume
MilliQ	18.9 ul
10x Pfu Buffer	3 ul
10mM dNTPs	0.6 ul
10uM F Primer	3 ul
10uM R Primer	3 ul
Template	1 ul
Pfu	0.5 ul
Total Volume	30 ul

Phusion

*Annealing temperatures for Phusion PCRs are calculated using the NEB Tm Calculator (http://tmcalculator.neb.com/#!/)

Reagent	Volume
MilliQ	19.9 ul
5x HF Buffer	6 ul
10mM dNTPs	0.6 ul
10uM F Primer	1 ul
10uM R Primer	1 ul
Template	1 ul
Pfu	0.5 ul
Total Volume	30 ul

Gel Extraction

Appropriately sized bands sliced from gel and DNA extracted according to Biobasic EZ-10 column protocol.

Ligation

Amount of Insert DNA to put in ligation calculated using this formula. All ligations were set up using a 4:1 insert to vector molar ratio with a maximum total of 100ng of DNA in a 10 ul reaction

$$ng\ insert\ DNA = \left(\frac{ng\ vector\ DNA * insert\ size\ (kb)}{vector\ size\ (kb)}\right) * \frac{4}{1}$$

Volumes of insert and vector DNA to add found using the quantities calculated using the above formula and measuring insert and vector concentration by nanodrop.

Reagent	Volume
MilliQ	To 10 ul total
NEB 10x Ligase buffer	1 ul
Insert DNA	X ul
Vector DNA	Y ul
T4 Ligase	0.5 ul
Total Volume	10 ul

Transformation

- 1.2 ul of the ligation mix added to 20ul of High efficiency NEBa competent cells
- Cells left on ice for 30 minutes

- Cells put in 42C water bath for 45 seconds
- Cells put back on ice for 5 minutes
- 400ul of SOC media added to cells
- Cells put in 37C shaking incubator for 1 hour
- 200 ul of cells plated on agar plates with appropriate antibiotic

Liquid Cultures

- 5ml LB culture tube spiked with 5ul of 1000x antibiotic stock
- Colonies or glycerol stocks touched with the end of a pipette tip
- Pipette tip swirled in culture tube. End of tube is flamed and capped
- Tube put in 37C shaking incubator for maximum 16 hours

Miniprep

Done according to instruction for Biobasic EZ-10 column instructions

Agarose Gel

For DNA fragments from 250bp to 8kb: samples ran on 1% agarose gel made with 1X TAE. Running buffer is 1X TAE. Ran for 30 minutes at 135v

Round the Horn Mutagenesis

This protocol is based off of the "Round the Horn Site-directed Mutagenesis" protocol by Sean Moore (http://openwetware.org/wiki/%27Round-the-horn_site-directed_mutagenesis)

Primer phosporylation

Reagent	Volume
MilliQ	32 ul
10x PNK buffer A	5 ul
25mM MgSO4	2 ul
100uM Primer	5 ul
20mM ATP	5 ul
PNK	1 ul
Total Volume	50 ul

Reaction is put in 37C incubator for 1 hour and heat killed at 95C for 5 minutes.

PCR reaction

Reagent	Volume
MilliQ	39 ul
10x Pfu Buffer	5 ul
10mM dNTPs	1 ul

10uM F Primer (phosphorylated)	1.5 ul
10uM R Primer (phosphorylated)	1.5 ul
Template	1 ul
Pfu	0.5 ul
Total Volume	50 ul

The standard PCR program described above is used.

DpnI digest

1ul of NEB DpnI enzyme added to PCR product. Incubated at 37C for 3 hours and heat killed at 80C for 20 minutes

Recircularization of Digested PCR product

Reagent	Volume
MilliQ	2.3 ul
NEB 10x Ligase buffer	0.5 ul
DpnI digest PCR product	2 ul
T4 Ligase	0.2 ul
Total Volume	5 ul

Exosome Prep

Generating exosome free media

Complete media is pre-spun to remove vesicles present in Fetal Bovine Serum. 12 ml of media is put into polycarbonate vessels and spun at 125,000g for 70 minutes.

Exosomes Isolation by Centrifugation

Media from cells collected, spun at 300g for 10 minutes to remove non-adherent cells, and filtered using a 200nm filter.

Filtered media spun at 125,000g for 70 minutes. Supernatant is removed and the pellet is resuspended in 1X PBS or 2% PFA depending on intended use.

Isolated exosomes stored at 4C.

Unless otherwise indicated, each of these protocols is carried out according to the standard protocols

April

Sunday, 6th

- Rehydration of K808000 and K112808 kit plate DNA
- Transformation of rehydrated kit plate DNA into compentent DH5a

Monday, 7th

- Colonies of K808000 and K112808 picked to start liquid cultures

Tuesday, 8th

- Minipreps of K808000 and K112808 cultures

Thursday, 10th

- Digest of K808000 and K112808 for assembly/confirmation
- Agarose gel of restrictions
- Gel extraction

Friday, 11th

- Ligation of K808000 and K112808 for killswitch production

Saturday, 12th

- Transformation of O/N ligation of K808000 and K112808

Sunday, 13th

- Colony PCR of K808000 and K112808 plates

Tuesday, 15th

- Ligation plates picked – pSB1C3 backbone

Wednesday, 16th

- Killswitch (K808000 + K112808) colonies miniprepped and sent for sequencing

May

Thursday, 15th

- Mutagenesis of K112808 for RNA-IN, Reconstitute primers, Reaction, DpnI digest

Friday, 16th

- Gel of PCR product and DpnI digest

Satuday, 17th

- DpnI transformed

Sunday, 18th

- plate did not grow, new mutagenesis method started

June

Wednesday, 4th

- Rehydration of K112808-RNA-OUT, transformed

Thursday, 5th

- RNA-OUT colonies picked and grown

Friday, 6th

- RNA-OUT glycerol stocked

Monday, 9th

- Making SOC media

July

Wednesday, 9th

- Lamp2B Synthesis resuspended and transformed into competent DH5a

Thursday, 10th

- Lamp2b plates picked

Friday, 11th

- Lamp2b cultures miniprepped and glycerol stocked
- Digests for Lamp2b and Clover assembly and RNA-OUT into pSB1C3 (overnight digests)

Saturday, 12th

- Digests ran on gel
- Digests gel extracted, nanodropped, and ligated overnight

Sunday, 13th

- Overnight ligations transformed

Monday, 14th

- *Plates of last night's ligation did not grow, changing ligation ratio to 4:1
- Lamp2b, clover, Digested, gel extracted and ligated today

Tuesday, 15th

- Ligation transformed

Wednesday, 16th

- P1010 rehydrated from kit plate and transformed into DH5a, ligations plates grew (Lamp2b-Clover and RNA-out pSB1C3) – miniprepped, digested, gelled, no good – start Lamp2b, Clover, RNA-out and Jo4450 from glycerol stock

Thursday, 17th

- P1010 plates did not grow, miniprepped overnight cultures, minipreps digested for assembly overnight

Friday, 18th

- Digests ran on gel, bands good, gel extracted and ligations set up.

Saturday, 19th

- Ligations transformed, p1010 rehydration retransformed.

Sunday, 20th

- All plates grew, picked colonies and grown overnight.

Monday, 21st

- Cultures miniprepped and digested for confirmation. Some worked for ligations, all p1010 were good, sequencing set up, cultures glycerol stocked

Thursday, 24th

- RTH mutagenesis to add S32-RNA-IN to the B0034 RBS, phosphorylated primers, PCR ran

Friday, 25th

- 5ul PCR saved, the rest had 1ul DpnI added and digested for 3 hours. Gel ran. See band at full plasmid size, ligation to recircularize set up

Saturday, 26th

- S32-b0034 ligation transformed. Attempted K112808 RNA-IN mutagenesis (QC) didn't work

Sunday, 27th

- RVG-HIS swap mutagenesis, phosphorylate primers, PCR reaction, gel and DpnI started, 1/10 dilution of lamp2b-clover transformed into rosetta2 DE3 cells

Tuesday, 29th

- TEV synthesis rehydrated and transformed. K112808 plates grew, picked and grown overnight

Wednesday, 30th

- TEV plates grew, picked and grown overnight, K112808 confirmation gel, some were good, successful looking preps sent for sequencing

Thursday, 31st

- TEV miniprepped, digested and confirmed as good.

August

Monday, 4th

- RTH K112808 mutagenesis, phosphorylate primer, PCR, DpnI

Tuesday, 5th

- Transformation K112808-IN

Wednesday, 6th

- K112808 plates grew, picked

Thursday, 7th

- Miniprepped K112808 in, sent for sequencing

Tuesday, 12th

- K112808in RTH Started again

Wednesday, 13th

- K112808in mutagenesis transformed

Thursday, 14th

- Picked the K112808 plates

Friday, 15th

- Miniprepped, gel confirmed and sent for sequencing

Saturday, 16th

- K112808in and K808000 assembly started, digest, gel, gel extraction and ligation

Sunday, 17th

- Ligation transformed

Monday, 18th

- Plates grew, picked and grown overnight,

Tuesday, 19th

- Miniprepped, gel looked good sent for sequencing

Saturday, 30th

- K808000+lyse-in and K808000 + lyse co transformed with RNA-OUT

September

Monday, 1st

- Plates grew, picked and grown overnight in the appropriate two antibiotics

Tuesday, 2nd

- Culture miniprepped, cut with e and s to see if both plasmids were there, they were not, picked other colonies

Wednesday, 3rd

- Miniprepped, not both. Cotransformation done again. Rehydrated K608351 heat sensitive promoter and transformed. Prespun media for exosomes collection

Thursday, 4th

- Picked K608351 plates. K145151 rehydrated from kit plate and transformed. Picked cotransformation plates

Friday, 5th

- K608351 cultures prepped and confirmed. Picked k145151 plates. Cotransformation cultures grew, miniprepped and digested again and only one plasmid again.

Saturday, 6th

- RTH mutagenesis of S32-RBS to add universal adaptor (RFC25 suffix). Picked K145151 colonies and grew overnight

Sunday, 7th

- RTH mutagenesis transformed, miniprep of K145151 and digest. Size is good. Miniprepped K145151

Monday, 8th

- S32-B0034 universal mutagenesis plates picked.

Tuesday, 9th

- S32-B0034-Uni cultures miniprepped and sequenced

Wednesday, 10th

- Exosomes isolates and fluorescence characterized.

Thursday, 11th

- Exosome samples characterized using confocal microscope and electron microscope

Monday, 15th

- RTH for s32-B0034-Uni started again. PCR to add RFC25 prefix and suffix to P1010 ccdB.

Tuesday, 16th

- RTH transformed . K105007 rehydrated and transformed

Wednesday, 17th

- Transformations grew, picked and grown overnight

Thursday, 18th

- Miniprepped RTH and send for sequencing.
- Miniprepped K105007 and started PCR to add NheI and PacI cut sites
- PCR to add NheI and PacI cut sites onto Lamp2b construct started

Friday, 19th

- K608351 and S32-RBS-Uni digested to begin assembly (K608351 upstream and S32-RBS-Uni downstream)
- Gel of digests ran and bands of interest gel extracted
- Extractions nandropped and ligated

Saturday, 20th

- * it looks like the replication origin on the lysis plasmids and the RNA-OUT plasmids are the same. This means they will both be competing for
- K808000-RNA-IN-K112808 and K808000-K112808 digested to be put into pSB2K3

October